Structural mechanism of RPA loading on DNA during activation of a simple pre-replication complex

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We report that during activation of the simian virus 40 (SV40) pre-replication complex, SV40 T antigen (Tag) helicase actively loads replication protein A (RPA) on emerging single-stranded DNA (ssDNA). This novel loading process requires physical interaction of Tag origin DNA-binding domain (OBD) with the RPA high-affinity ssDNA-binding domains (RPA70AB). Heteronuclear NMR chemical shift mapping revealed that Tag-OBD binds to RPA70AB at a site distal from the ssDNA-binding sites and that RPA70AB, Tag-OBD, and an 8-nucleotide ssDNA form a stable ternary complex. Intact RPA and Tag also interact stably in the presence of an 8-mer, but Tag dissociates from the complex when RPA binds to longer oligonucleotides. Together, our results imply that an allosteric change in RPA quaternary structure completes the loading reaction. A mechanistic model is proposed in which the ternary complex is a key intermediate that directly couples origin DNA unwinding to RPA loading on emerging ssDNA.

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Introduction

Replication protein A (RPA) is a conserved eukaryotic single-stranded DNA (ssDNA)-binding protein that was discovered as an essential factor in the cell-free replication of simian virus 40 (SV40) DNA (Wold, 1997; Iftode *et al*, 1999; Bochkarev and Bochkareva, 2004). A large body of evidence

now demonstrates that RPA is an obligatory participant in most eukaryotic DNA processing pathways, from DNA replication, repair, and recombination through somatic hypermutation in lymphocytes and DNA damage signaling (Zou and Elledge, 2003; Chaudhuri *et al*, 2004; Stauffer and Chazin, 2004).

RPA is a highly flexible modular protein composed of three subunits (RPA70, RPA32, RPA14) that are stably associated with each other (Figure 1). Three-dimensional structures of each of the seven individual domains and several subassemblies are now available, but the quaternary structure(s) of RPA remains unknown (Bochkarev and Bochkareva, 2004; Stauffer and Chazin, 2004). Four of the domains (DNA-binding domains (DBDs) A–D) bind to ssDNA with decreasing affinity from A to D (Figure 1). RPA binding to ssDNA occurs sequentially and with a defined polarity, beginning with DBD-A and -B at the 5′ end, yielding three different states that occlude 8–10, 12–23, or 28–30 nucleotides (de Laat *et al*, 1998; Iftode and Borowiec, 2000; Bastin-Shanower and Brill, 2001; Arunkumar *et al*, 2003; Wyka *et al*, 2003). The three states of RPA are thought to coexist in solution, in dynamic equilibrium with each other (Blackwell *et al*, 1996). Studies of free RPA70AB (residues 181–422) reveal that the linker peptide between domains A and B is flexible (Arunkumar *et al*, 2003), whereas RPA70AB bound to an 8-nucleotide ssDNA adopts a structure with domains A, B, and the linker in a fixed orientation (Bochkarev *et al*, 1997; Bochkareva *et al*, 2001; Arunkumar *et al*, 2003).

RPA also interacts directly with and modulates the activity of a large and growing number of factors that are required for proper processing of ssDNA (Iftode *et al*, 1999; Fanning *et al*, 2006). The binding sites for these proteins have been mapped to regions in the RPA70N, 70A, 70B, and 32C domains (Figure 1). Notably, many of the DNA processing factors are, like RPA, modular multi-domain proteins, and in most instances, their interactions with RPA involve multiple contact points between two or more domains.

The viral replicative DNA helicase, SV40 T antigen (Tag), binds to RPA via its origin DNA-binding domain (OBD, residues 131–259) and this interaction is essential for SV40 DNA replication (Dornreiter *et al*, 1992; Melendy and Stillman, 1993; Weissbart *et al*, 1998). The Tag-binding regions of RPA have been mapped to RPA70 residues 169–327 (Braun *et al*, 1997) or RPA70A (Loo and Melendy, 2004; Park *et al*, 2005), and RPA32C (Lee and Kim, 1995). Although the physical interaction of Tag-OBD with RPA70 remains poorly understood, an important step forward was made possible by construction of a model of Tag-OBD and RPA32C from extensive NMR data (Arunkumar *et al*, 2005).

An understanding of how RPA functions in the progression of ssDNA processing pathways remains elusive. One proposal is that processing proteins successively compete with each other for RPA, allowing them to switch places on DNA as the

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Figure 1 Domain organization of RPA with Tag-binding regions shaded. Rectangles depict OB-folds, of which four are ssDNA-binding domains (DBDs; A–D); an oval indicates the winged-helix–turn–helix of RPA32C (Mer *et al*, 2000); a circled P depicts the phosphorylated region of RPA32. A triple arrow symbolizes the association of subunits through a three-helix bundle (Bochkareva *et al*, 2002), and hexagons denote Tag hexamers.

pathway progresses toward completion (Yuzhakov *et al*, 1999). This mechanism, termed hand-off, correlates with the increasing affinity of proteins for RPA during lagging strand SV40 DNA replication *in vitro* (Yuzhakov *et al*, 1999). There is also evidence for a mechanism involving protein-mediated remodeling of RPA, from an extended conformation into a compact conformation that more readily dissociates from ssDNA, which allows incoming proteins to access ssDNA. For example, we recently demonstrated that Tag binding to RPA32C facilitates replacement of RPA on ssDNA by DNA polymerase alpha-primase to promote primer synthesis, and proposed that this transition is based on the ability of Tag to bind and remodel the ssDNA-binding mode of RPA (Arunkumar *et al*, 2005).

The ability of RPA-binding proteins to facilitate its displacement from ssDNA led us to consider whether RPA freely diffuses onto ssDNA or whether DNA processing proteins actively load RPA on ssDNA. Here, we demonstrate that the Tag double hexamer, which serves as a simple pre-replication complex on the viral origin, selectively loads human RPA on the emerging ssDNA at the origin. To define the structural basis for this loading reaction, we map the physical interaction surfaces of Tag and RPA70AB in detail and show that Tag forms a ternary complex with RPA70 domains A and B bound to a minimal ssDNA-binding site of 8 nucleotides. However, in the presence of a 30-nucleotide oligomer, Tag was found to dissociate from the ternary complex. Our evidence suggests that the ternary complex initially couples activation of SV40 origin unwinding by Tag helicase with RPA loading onto ssDNA, and then dissociates to enable RPA binding in an extended mode to the full ssDNA site.

Results

Selective loading of human RPA during unwinding of the SV40 replication origin

To determine whether origin DNA unwinding by Tag double hexamer is coupled to RPA recruitment and concomitant loading onto ssDNA, we asked whether Tag would selectively load human RPA onto emerging ssDNA in the presence of a competing ssDNA-binding protein that supports origin unwinding but not later steps in SV40 replication. Yeast RPA was chosen as the competitor in this experiment because it binds ssDNA with an affinity similar to human RPA (Figure 2A and B) and supports Tag-catalyzed unwinding of SV40 origin

DNA, but it does not bind to Tag and does not support primer synthesis in SV40 replication (Brill and Stillman, 1989; Melendy and Stillman, 1993; Iftode and Borowiec, 1997; Sibenaller *et al*, 1998; Arunkumar *et al*, 2005). We reasoned that if yeast RPA can compete with human RPA for the emerging ssDNA, it should inhibit replication of the template DNA in a concentration-dependent manner.

To test this prediction, initiation of SV40 replication was monitored in a reaction containing supercoiled origin DNA, and purified proteins Tag, DNA polymerase alpha-primase, topoisomerase I, a limiting amount of human RPA, ribo- and deoxyribonucleotides, and radiolabeled dTTP. In this reaction, a low level of initiation (Figure 2D, lane 1) was detected, although clearly above that in negative control reactions (lanes 9 and 10). Additional human RPA stimulated robust initiation (lanes 2–4), confirming that the lowest level of human RPA was sub-saturating. As expected, no replication products were detected in a reaction that contained yeast RPA in place of human RPA (lane 11). Moreover, addition of yeast RPA in up to a five-fold excess over the human RPA failed to reduce the initiation activity of human RPA (lanes 5–8). In fact, we note a weak stimulation that, based on the absence of activity in lane 11, appears to be nonspecific. Together, these results suggest that Tag selectively loaded human RPA onto ssDNA during origin unwinding, which prevents yeast RPA from gaining access to the template.

To confirm this interpretation, SV40 initiation was tested using limiting human RPA in the presence of increasing amounts of a chimeric RPA hRPAy32C, in which the yeast RPA32C domain replaced the human RPA32C domain. This chimeric RPA binds to ssDNA (Figure 2A and C) and to Tag, but does not support Tag-mediated primer synthesis (Arunkumar *et al*, 2005). As in Figure 2D, the low initiation activity with limiting human RPA (Figure 2E, lane 1) was stimulated by additional human RPA (lanes 2–4). However, in contrast to the results with excess yeast RPA (Figure 2D), increasing amounts of chimeric RPA inhibited initiation in a dose-dependent manner (Figure 2E, lanes 5–8), almost down to the level detected in a control reaction with chimeric RPA alone (lane 11). These results implicate physical interactions of Tag with the RPA70 subunit in selectively loading RPA onto ssDNA during unwinding. Taken together, the results in Figure 2 strongly suggest that origin unwinding is coupled with RPA loading directly onto the emerging ssDNA.

Domain mapping of interactions between Tag and RPA

The results in Figure 2 suggest that physical interaction between Tag and RPA could play an important role in coupling origin unwinding with RPA loading. Detailed mapping of the interactions between Tag and RPA70 was performed to confirm which domains of Tag and RPA70 are involved. Our first approach involved pull-down assays in which a constant amount of full-length Tag or GST-fused Tag-OBD was titrated with purified intact RPA or RPA70AB under buffer conditions similar to those used for cell-free SV40 DNA replication (Supplementary Figure 1). RPA binding to purified Tag was easily detectable with small amounts of RPA (Supplementary Figure 1B). When the same amount of Tag beads was incubated with RPA70AB, which lacks the known RPA32C interaction region, about eight-fold higher molar amounts were required to detect a level of binding comparable to that obtained with RPA heterotrimer (Supplementary

Figure 2 Tag interaction with RPA70AB actively loads human RPA onto ssDNA during initiation of SV40 DNA replication. **(A)** Purified hRPA, yRPA, and hRPAy32C chimera were visualized by SDS-PAGE and Coomassie stain. PM, protein mass marker. **(B, C)** Filter binding assays were used to compare the activity of the indicated RPAs in binding to radiolabeled dT₃₀. **(D, E)** SV40 monopolymerase assays were performed with a suboptimal amount of human RPA (0.2 mg) (lane 1) supplemented with up to five-fold greater amounts of human RPA (lanes 2–4) and either **(D)** yeast RPA or **(E)** hRPAy32C (lanes 5–8) as indicated. Reaction products were visualized by denaturing gel electrophoresis and autoradiography (left), and quantified by scintillation counting (right). Negative control reactions lacked Tag, polymerase alpha-primase, or human RPA as indicated (lanes 9–11).

Figure 1C). Consistent with this observation, GST-OBD beads bound full-length RPA quite well, and about eight-fold greater molar amounts of RPA70AB were required to detect comparable binding (Supplementary Figure 1D and E). These data confirm that RPA binds to Tag-OBD through RPA70AB, in addition to RPA32C, and that both interactions contribute to the overall binding affinity.

A second approach to characterize RPA70AB interactions with Tag-OBD involved proteolysis protection assays. In RPA70AB, the flexible linker between the two high-affinity ssDNA-binding domains A and B is sensitive to proteolysis (Gomes and Wold, 1996; Gomes *et al*, 1996). RPA70AB binding to ssDNA fixes the relative positions of the two

domains and protects the linker against digestion (Gomes *et al.*, 1996; Bochkarev *et al.*, 1997). To gain insight into the interaction of RPA70AB with Tag-OBD, limited proteolysis of RPA70AB was carried out in the presence and absence of OBD. Without OBD, the linker between RPA70A and -B was almost completely cleaved after digestion for 2 h (Figure 3A). In contrast, intact RPA70AB remained detectable for at least 15 h in the presence of OBD (Figure 3B). The stabilization of the linker strongly resembled that observed for RPA70AB digested in the presence of ssDNA (dC₈) (Figure 3C). These observations indicate that like ssDNA, OBD binds and stabilizes RPA70AB, but do not reveal whether the OBD binds to the same surface of RPA as ssDNA or a different one.

Figure 3 Tag-OBD protects RPA70AB from proteolytic digestion. RPA70AB was incubated with trypsin for the indicated time periods, either alone (A) or in the presence of Tag-OBD (B) d(C)₈ (C), or d(C)₈ and Tag-OBD (D). Digestion products were visualized by SDS-PAGE and Coomassie staining.

However, Figure 3D shows that RPA70AB was almost completely resistant to trypsin proteolysis in the presence of both ssDNA and OBD. The fact that ssDNA and OBD together stabilized RPA70AB more effectively than did either alone implies that a stable ternary complex is formed with discrete binding sites for each component.

RPA70AB binds ssDNA and Tag-OBD at distinct sites

In order to determine the structural basis for the proposed coupling of origin unwinding to active RPA loading onto emerging ssDNA, heteronuclear NMR was used to directly map the Tag-OBD interacting surface of RPA70AB. The strategy involved monitoring perturbations of NMR signals as Tag-OBD is titrated into a solution of RPA70AB. This approach has proven extremely valuable for mapping binding surfaces because the NMR chemical shift for each nucleus is extremely sensitive to its electronic environment. While perturbations will arise from direct binding as well as allosteric structural changes, binding sites can often be distinguished because a series of spatially proximate residues that form a contiguous surface is discernable from the ensemble of chemical shift data.

The experiments were performed by monitoring ¹⁵N-¹H HSQC spectra of ¹⁵N-enriched RPA70AB as unlabeled Tag-OBD was titrated because this allowed RPA70AB signals to be cleanly discriminated from those of Tag-OBD. A single set of signals was observed at all points during the titration, which indicates that RPA70AB is in fast exchange between its free and bound states, consistent with relatively weak binding in the micromolar range. Figure 4A shows the overlay of a portion of an HSQC spectrum of free RPA70AB and one obtained in the presence of 2 molar excess of Tag-OBD. Although most of the peaks were not affected by the presence of OBD, a subset of the peaks progressively moved or broadened as the titration proceeded. Residues that displayed readily discernible changes in NMR chemical shift are labeled in the inset of Figure 4A. Mapping of the residues perturbed upon titration with Tag-OBD on the structure of RPA70AB revealed a contiguous surface along the linker and into both domains (Figure 4B). To confirm that this is a physically

realistic surface for Tag-OBD binding, the RPA70AB and Tag-OBD structures were inspected to confirm that Tag-OBD is large enough to span across the linker and interact with both the A and B domains. Overall, these results imply that Tag-OBD binds to RPA70AB at a site that is remote from the ssDNA-binding sites.

To obtain further insight into the coupling of the binding of ssDNA and Tag-OBD to RPA70AB, additional NMR spectra were recorded for ¹⁵N-enriched RPA70AB in the binary complex with ssDNA and the ternary complex with OBD and ssDNA (Figure 4C). Remarkably, the overall quality of the spectra was better for the larger ternary complex than for either of the binary complexes, a reflection of an increased structural stability of the ternary complex. Comparison of these spectra revealed three important points. First, the changes induced in the NMR spectrum by binding of ssDNA were mostly distinct from those induced by binding of Tag-OBD. For example, the changes in chemical shifts for D291 and Q299 are observed only when OBD is added to the solution, regardless of whether or not ssDNA is present (Figure 4C). Second, in addition to perturbations of RPA70AB chemical shifts, new peaks appear in the spectrum, which can be attributed to resonances from residues that are stabilized upon binding. Third, the spectrum of the ternary complex of RPA70AB, OBD, and ssDNA contains all of the 'new' peaks that appear in the spectra of the binary complexes, as well as a very small number of additional peaks. Thus, in addition to revealing that Tag-OBD binds to a surface of RPA70AB that is remote from the ssDNA-binding site, these observations provide evidence for the energetic coupling of the binding of two different ligands (ssDNA and Tag-OBD) to RPA70AB, which suggests the possibility of function through an allosteric structural mechanism (*vide infra*).

Tag-OBD associates with RPA70AB through electrostatic interactions

In order to deepen our understanding of the structural basis for the interaction between RPA70AB and Tag-OBD, the NMR-based strategy was used to map the region of Tag-OBD that binds to RPA70AB. To this end, ¹⁵N-enriched Tag-OBD was

Figure 4 Structural mapping of RPA70AB-binding site for Tag-OBD. (A) Chemical shift mapping of Tag-OBD-binding site on RPA70AB. Overlay of ^{15}N - ^1H HSQC spectra of ^{15}N -enriched RPA70AB in the absence (black) and presence of Tag-OBD (red). The inset shows some of the residues that are perturbed or broadened upon interaction with Tag-OBD. (B) Molecular surface of RPA70AB with residues whose chemical shifts are perturbed upon binding of Tag-OBD colored in blue (strongest effects) and green (other significant effects). (C) Formation of a stable ternary complex of RPA70AB with d(C)₈ and Tag-OBD. The left panel shows a region from the ^{15}N - ^1H HSQC spectra of the binary complex of ^{15}N -enriched RPA70AB with d(C)₈ in the absence (black) and presence of Tag-OBD (red). The right panel shows a region from the ^{15}N - ^1H HSQC spectra of the binary complex of ^{15}N -enriched RPA70AB with Tag-OBD in the absence (black) and presence of d(C)₈ Tag-OBD (red).

titrated with unlabeled RPA70AB and chemical shift perturbations were monitored (Figure 5A). The most significant effect detected was the perturbation of residues from three regions that together form a contiguous binding surface on Tag-OBD (F151-T155, F183-H187, H203-A207) (Figure 5B, circle). This surface has a significant basic character arising primarily from three prominent surface residues: R154, R202, and R204.

These results, combined with the analysis of the RPA70AB-binding site, indicate that there is a significant electrostatic component to the interaction of Tag-OBD with RPA70AB. In particular, the basic residues on the Tag-OBD-binding surface

are complemented by the highly acidic nature of the RPA70AB-binding surface, in which residues E290, D291, D292, D404, and D407 form a dense negatively charged surface on the opposite side of RPA70AB from the ssDNA-binding surface (Figure 5C). To verify the importance of the electrostatic complementarity observed in the RPA70AB-Tag-OBD-binding interface, charge reversal mutations were designed in Tag-OBD at the RPA-binding interface with the goal of weakening the interaction with RPA70AB. Indeed, RPA70AB binding of purified full-length Tag containing the R154E mutation was 8- to 10-fold weaker than of wild-type Tag (Figure 5D, lanes 3–6; Figure 5E), confirming the importance of the electrostatic character of the interaction surface. As a control, the mutant Tag-OBD was subjected to biophysical/structural analysis, which showed that the reduction in binding affinity did not arise from perturbation of the structure (Supplementary Figure 2).

Physical interaction with Tag-OBD stimulates ssDNA binding of RPA

The finding that Tag-OBD and ssDNA bind to different sites in RPA70AB led us to ask whether full-length Tag can also form a ternary complex with trimeric RPA and ssDNA. To examine this possibility, RPA complexes were pre-formed with Tag adsorbed to antibody beads, and after unbound RPA was removed, ssDNA was titrated into the complexes. The lengths of ssDNA chosen for this experiment (8, 15, or 30 nucleotides) correspond to those bound by RPA in its three different binding modes (see Introduction). The complexes were incubated with ssDNA for 1 h and then analyzed by SDS-PAGE and Western blotting to detect bound RPA (Figure 6A). In the absence of ssDNA, RPA remained stably bound to the Tag beads for at least 1 h after removal of the free RPA (lanes 3). When the Tag-RPA complexes were exposed to an 8-nucleotide ssDNA (upper panel) or, as a negative control, to duplex DNA (not shown), the complexes remained stable. In the presence of a 15-mer, most of the RPA remained bound to the Tag beads except when exposed to the highest amounts of ssDNA (middle panel, lanes 8 and 9). Remarkably, in the presence of a 30-mer, most of the bound RPA was dissociated from Tag (lower panel, lanes 4–9). These findings indicate that the length of ssDNA bound to RPA affects its ability to exist in a stable complex with Tag. One possible interpretation of the data is that RPA in its compact binding mode with an 8-mer forms a stable ternary complex with Tag, but that RPA in its extended binding mode with a 30-mer binds more weakly to Tag, leading to its dissociation from Tag. This correlates with the greater affinity of RPA for a 30-mer than for shorter oligonucleotides.

To gain further insight into the interaction of RPA with ssDNA in the presence and absence of Tag, electrophoretic mobility shift assays were performed. In the absence of Tag, RPA bound to radiolabeled ssDNA (dT₃₀) with the expected 1:1 stoichiometry of RPA:ssDNA (RPA I) (Figure 6B, lanes 1, 4, and 7). Importantly, when Tag was added to the same amount of RPA that had been pre-incubated with labeled dT₃₀, the stoichiometry remained 1:1 but the abundance of the RPA:ssDNA complex clearly increased (lanes 2, 3, 5, and 6). Hence, Tag stimulated RPA binding to the 30-nucleotide ssDNA, even though there is no evidence of Tag being present in the complex along with RPA and ssDNA.

Figure 5 Structural mapping of Tag-OBD-binding site on RPA70AB. **(A)** Chemical shift perturbation analysis of the Tag-OBD-binding site on RPA70AB. Overlay of ^{15}N - ^1H HSQC spectra of ^{15}N -enriched Tag-OBD in the absence (black) and presence of RPA70AB (red). A expansion of a small region is shown, highlighting some of the residues that are perturbed or broadened upon interaction with RPA70AB. **(B, C)** Molecular surface diagrams of the electrostatic potential of Tag-OBD and RPA70AB, respectively, with blue for positive charge and red for negative charge. The large yellow circle in (B) highlights the contiguous binding region composed of regions F151-T155, F183-H187, and H203-A207. The three prominent Arg residues providing the bulk of the basic character of this region are labeled. The small yellow circles in (C) highlight the six acidic residues that provide the acidic character to the Tag-OBD-binding site on RPA70AB. The ssDNA in the RPA70AB structure is colored yellow. **(D)** A charge reversal mutation in Tag-OBD reduces Tag binding to RPA70AB. Wild-type (lanes 1, 3, 4) and R154E mutant Tag (lanes 2, 5, 6) adsorbed to antibody beads were incubated with 5 or 10 μg of RPA70AB as indicated. Proteins bound to the beads were separated by SDS-PAGE and visualized by Western blotting with Mab70C against RPA (top panel) or Pab101 against Tag (lower panel). Antibody beads lacking Tag did not bind RPA70AB (lane 7). Lane 8 shows 200 ng of input RPA70AB. **(E)** Quantification of bound RPA70AB in lanes 3–6 of (D) after subtraction of background in lane 7.

A novel slower migrating, diffuse species was observed at the highest concentration of RPA when Tag was present (RPAIL; Figure 6B, lanes 8 and 9). To characterize the composition of this complex, the mobility shift experiment was repeated in the presence of monoclonal antibody against RPA or Tag. When a saturating amount of RPA was incubated with radiolabeled ssDNA (Figure 6C, lanes 2 and 3), addition of Tag was seen to lead to the formation of a slowly migrating ssDNA complex (RPAIL) (lane 3). When purified monoclonal antibody against the extreme C-terminus of Tag was titrated into the binding reaction, the mobility of the ssDNA complex remained unchanged (lanes 4–6). Two other antibodies that recognize epitopes remote from the Tag-OBD and do not interfere with RPA:Tag complex formation (Weissart *et al*, 1998) yielded identical results (not shown). These results indicated that Tag is not present in RPAIL. The mobility of the RPAIL complex was also unaffected by the addition of control antibody against influenza hemagglutinin (lanes 7–9), but was supershifted to a slower mobility in the presence of antibody against RPA32C (lanes 10 and 11). We conclude that the RPAIL complex observed at high RPA concentrations is not a ternary complex with Tag. We believe that RPAIL may contain two or three RPA molecules bound to the 30-mer in a compact binding mode (Blackwell *et al*, 1996).

As Tag did not appear to form a stable ternary complex with RPA and a 30-mer ssDNA, we asked whether direct

physical interaction between Tag and RPA was necessary to stimulate ssDNA binding to RPA. To address this question, additional mobility shift assays were carried out with RPA from budding yeast, which binds ssDNA but not Tag (Melendy and Stillman, 1993). In these experiments, ssDNA binding of yeast RPA was unaffected by Tag (Figure 6D, lanes 2–7), arguing that at least a transient Tag interaction with RPA is required to enhance RPA binding to a 30-mer. To further assess the role of Tag–RPA interaction in facilitating RPA binding to ssDNA, the previously discussed mutant Tag (R154E) with reduced affinity for RPA70AB (Figure 5D) was tested in mobility shift experiments (Figure 6E). Tag R154E retained the ability to stimulate RPA binding to dT₃₀, but its activity was clearly reduced (Figure 6E, compare lanes 3 and 6). The results in Figures 6D and E indicate that the ability of Tag to facilitate RPA binding to a 30-nucleotide ssDNA correlates with Tag–RPA affinity.

Discussion

We have shown here for the first time that during activation of a simple model pre-replication complex, origin DNA unwinding is directly coupled to RPA loading on the emerging ssDNA, and that physical interaction of the pre-replication protein Tag with RPA70AB, but not RPA32C, is required for this novel coupling (Figure 2). The interaction surfaces of

Figure 6 Transient Tag binding to RPA facilitates ssDNA binding of RPA. (A) Tag bound to Pab101-protein G beads was incubated with RPA (8.6 pmol) or without RPA (con). After washing the beads, increasing amounts of oligonucleotides dT₃₀, dT₁₅, or dT₈ (0, 1, 2, 4, 9, 17, or 34 pmol) were added. After 1 h, the amount of RPA that remained bound to Tag was visualized by SDS-PAGE and Western blot with anti-RPA70 antibody. Input: 5% of the RPA added to samples. (B) RPA in the indicated amounts (pmol) was pre-incubated with ~3 pmol of radiolabeled dT₃₀ for 10 min at 25°C and then, after addition of the indicated amounts of Tag (pmol of hexamer), for another 15 min at 37°C. Protein-DNA complexes were detected by native gel electrophoresis and autoradiography. The migration of Tag-dT₃₀ complex (Supplementary Figure 3) is indicated. W, wells of the gel. (C) RPA (9 pmol) was incubated with ~3 pmol of dT₃₀, followed by addition of 3.5 pmol of Tag hexamer and, after 5 min, monoclonal antibody (0.5, 2.5, or 5 µg) against Tag (T), influenza hemagglutinin (N), or RPA32C (R). Complexes were visualized as in (B). (D) The indicated amounts (pmol) of yeast RPA (yRPA) were incubated with ~3 pmol of radiolabeled dT₃₀ in the presence of Tag hexamer (pmol) as indicated. Complexes were analyzed as in (B). (E) Increasing amounts (0.5 or 1 pmol of hexamer) of wild-type (lanes 3 and 4) or mutant R154E Tag (lanes 6 and 7) were added to RPA (2 pmol) that had been pre-incubated with radiolabeled dT₃₀ as indicated, and protein-DNA complexes were analyzed by native gel electrophoresis and autoradiography as in (B).

Tag-OB and RPA70AB have been mapped, revealing a strong electrostatic component. Formation of a stable ternary complex composed of RPA, an 8-mer ssDNA, and Tag has been demonstrated (Figures 3–6). However, RPA binding to a 15- to 30-mer ssDNA was shown to lead to dissociation of Tag from the ternary complex (Figure 6), completing the RPA loading process.

The structural perspective provided in this study significantly advances our understanding of the complex interplay between RPA and Tag during DNA unwinding. The ability of RPA to bind simultaneously to Tag and an 8-mer ssDNA in a ternary complex demonstrates that Tag and ssDNA do not compete directly for RPA. Our evidence suggests that dissociation of Tag from the ternary complex in the presence of 15- to 30-nucleotide ssDNA involves allosteric remodeling of RPA quaternary structure from a compact 8-nucleotide binding mode into an extended ssDNA binding mode that weakens Tag binding. The precise nature of these conformational changes is currently under investigation.

The ternary complex: a coupling device for protein-mediated RPA loading during DNA unwinding

Our new results suggest a simple two-step model for Tag-mediated RPA loading on ssDNA as it emerges from the active helicase (Figure 7A). Once the Tag hexamer is active, it moves

3'-5' on ssDNA, displacing the complementary strand. Based on the recent crystal structure of the closely related papillomavirus E1 helicase domain in complex with ssDNA (Enemark and Joshua-Tor, 2006), the displaced strand would emerge close to the Tag-OB (Figure 7A, top). Our model suggests that the basic RPA70AB-binding surface of a Tag-OB would be exposed to the exterior as the helicase moves, positioning it to bind RPA. An exposed basic surface of Tag-OB was recently noted in the crystal structure of hexameric Tag-OB in an open spiral (Meinke *et al*, 2006). Based on our findings, formation of the ternary complex would occur upon the extrusion of 8–10 nucleotides of ssDNA. Continued helicase action (~200 nucleotides/min) (Murakami and Hurwitz, 1993) would rapidly generate a 30-nucleotide stretch of ssDNA, allowing RPA to bind in an extended ssDNA binding mode that weakens binding to Tag-OB and leads to Tag release (Figure 7A, bottom). This simple model depicts an initial and a final step in loading each RPA molecule, but is not meant to rule out the possible existence of intermediate steps.

During initiation of SV40 DNA replication (Bullock, 1997; Simmons, 2000; Fanning and Pipas, 2006), a similar structural mechanism may couple origin DNA unwinding with RPA loading on the emerging ssDNA, as depicted in Figure 7B. SV40 DNA replication begins with the assembly of a simple

Figure 7 Proposed mechanism for coupling activation of the SV40 pre-replication complex with RPA loading on ssDNA. (A) An active Tag hexamer (OBD and N-terminus in gold; helicase domains in turquoise) translocating 3' to 5' on the red strand (dotted line) and displacing the blue strand is proposed to form a ternary complex, in which Tag-OBD binds to RPA70AB (orange) associated with 8–10 nucleotides of ssDNA. As more DNA is unwound, RPA extends into the 30-nucleotide binding mode on ssDNA and releases Tag-OBD. (B) Tag monomers recognize four specific binding sites (black arrows) in the origin DNA, nucleating double hexamer assembly and inducing an ssDNA bubble at the flanking EP sequence and distortion at AT. The depicted path of DNA through the Tag double hexamer is speculative (Gai *et al*, 2004b; Li *et al*, 2003; Valle *et al*, 2006). (C) Remodeling of the Tag N-terminus and Tag-OBD (Meinke *et al*, 2006; Valle *et al*, 2006) in the presence of human RPA is proposed to facilitate formation of a ternary complex composed of RPA70AB bound to an ssDNA bubble and to a basic OBD surface exposed on the exterior of each hexamer, analogous to that shown in (A). As the helicase translocates (dotted lines) and more ssDNA emerges, RPA would extend into the 30-nucleotide binding mode on ssDNA (orange arrows) and dissociate from Tag, completing the loading cycle.

pre-replication complex, the Tag double hexamer, in the presence of ATP at the central palindrome of the viral origin, nucleated by sequence-specific contacts of two subunits in each hexamer with the four pentanucleotides in the palindrome (arrows in Figure 7B). Assembly of the double hexamer is accompanied by remodeling of contacts between Tag subunits, both in the helicase domain and in the N-terminal and OBD domains (Smelkova and Borowiec, 1998; Weissbart *et al*, 1999, 2004; Li *et al*, 2003; Gai *et al*, 2004a,b; Meinke *et al*, 2006; Valle *et al*, 2006). This remodeling gives rise to distortion of the duplex DNA, generating an 8-nucleotide bubble in the EP region and an untwisted AT region at the opposite end of the origin (Figure 7B) (Borowiec and

Hurwitz, 1988). The single-stranded bubble thus represents an early intermediate in the unwinding reaction catalyzed by the double hexamer and marks the origin of bidirectional replication from which the two leading strands initiate and diverge (Hay and DePamphilis, 1982).

Interestingly, the ability of heterologous ssDNA-binding proteins to bind to a synthetic 8-nucleotide ssDNA-bubble substrate correlates with their ability to support SV40 origin DNA unwinding by Tag (Iftode and Borowiec, 1997, 1998), suggesting a possible role for RPA binding to such a bubble during unwinding. We propose that origin distortion leads to extrusion of an ssDNA bubble near the OBDs and to exposure of at least one OBD surface from each hexamer (Gai *et al*, 2004b; Meinke *et al*, 2006; Valle *et al*, 2006), enabling the formation of a ternary complex of RPA and Tag-OBD with the ssDNA bubble (Figure 7C). Thus, as noted for RPA loading on emergent ssDNA from the active helicase, the ternary complex would physically couple origin unwinding with RPA loading. As origin DNA unwinding progresses, more ssDNA would be extruded from the double hexamer (Wessel *et al*, 1992; Murakami and Hurwitz, 1993; Fanning, 1994; Smelkova and Borowiec, 1998; Alexandrov *et al*, 2002). Repetition of the unwinding and RPA loading cycle to generate enough template for replisome assembly would set the stage for Tag-mediated RPA displacement and DNA polymerase α -primase loading to initiate leading strand synthesis (Arunkumar *et al*, 2005).

Although this model is speculative, it allows several previously unexplained observations to be rationalized. For example, excess yeast RPA is unable to compete effectively with human RPA in SV40 initiation (Figure 2D), which can be explained by the fact that the ssDNA-bubble complex bound to yeast RPA could not be stabilized in a ternary complex with Tag. Our model also offers an explanation for the previously puzzling observation that a monoclonal antibody against RPA70 (Mab70C) inhibits SV40 origin DNA unwinding even though Mab70C does not inhibit ssDNA binding of RPA (Kenny *et al*, 1990). Notably, Mab70C binds to the same region of RPA70AB that interacts with Tag-OBD (Gomes and Wold, 1996) and presumably inhibits ternary complex formation with Tag, thereby inhibiting origin unwinding.

Our proposal of a ternary complex of RPA70AB, Tag, and an 8-mer as the initial step in protein-mediated RPA loading on ssDNA is fully consistent with the sequential binding mechanism of RPA domains A–D to ssDNA that was proposed for purified RPA (Bochkareva *et al*, 2002; Arunkumar *et al*, 2003; Wyka *et al*, 2003). Protein-mediated RPA loading on emerging ssDNA offers obvious advantages over diffusion-mediated loading on pre-existing ssDNA, as the ssDNA would be continuously protected from nucleases and potential hairpin formation from the time of its creation until the restoration of the duplex structure. Moreover, protein-mediated RPA loading on ssDNA offers the potential to create a regular, ordered array of RPA on ssDNA that may facilitate subsequent DNA processing more effectively than diffusion-mediated RPA loading on free ssDNA. The structural resemblance of the simple SV40 pre-replication complex to MCM double hexamers in the more elaborate eukaryotic pre-replication complex (Forsburg, 2004; Sclafani *et al*, 2004) is intriguing in this context. Our results provide strong motivation to determine whether DNA unwinding at chromosomal pre-replication complexes may also be coupled with RPA loading.

Materials and methods

Protein and DNA

Human RPA70AB was expressed and purified as described (Arunkumar *et al*, 2003). Tag_{131–259} (Tag-OBD), and recombinant trimeric human RPA, yeast RPA, and hRPAy32C chimera were expressed and purified as described (Arunkumar *et al*, 2005). SV40 Tag, DNA polymerase alpha-primase, and topoisomerase I were prepared as described (Ott *et al*, 2002). Monoclonal antibodies Pab101 against Tag (residues 696–708) (Weissbart *et al*, 2004) and 70C and 34A against hRPA (Kenny *et al*, 1990) were purified as described. HPLC pure oligo dT₃₀, dT₁₅, and dT₈ were purchased from Integrated DNA Technologies (Coralville, IA).

Uniformly enriched ¹⁵N and ¹³C,¹⁵N samples were prepared in minimal medium containing 1 g/l ¹⁵NH₄Cl (CIL Inc.) and 2 g/l unlabeled or [¹³C₆]glucose (CIL Inc.), respectively. HPLC pure ssDNA, d(C)₈, was purchased from Midland Certified Co. (Midland, TX) and used without further purification.

NMR spectroscopy

All NMR experiments were performed on Bruker Avance spectrometers operating at 600 and 800 MHz. The buffer that was used for all the NMR experiments was 20 mM Tris-d₁₁ HCl containing 50 mM KCl, 10 mM MgCl₂, 2 mM DTT, and 0.01 % NaN₃ at pH 7.2. All NMR experiments were recorded at 25°C. Two-dimensional, gradient-enhanced HSQC and TROSY-HSQC spectra were recorded with 4K complex data points in the ¹H and 200 complex points in ¹⁵N dimension. All NMR spectra were processed and analyzed using Felix 2000 (Accelrys Inc., San Diego, CA). Complete backbone and side-chain assignments for RPA70AB and Tag-OBD are reported elsewhere (Luo *et al*, 1996; Bhattacharya *et al*, 2004). Structures were visualized and figures were generated using MOLMOL (Koradi *et al*, 1996).

Limited proteolysis

Limited proteolysis of RPA70AB was carried out in the presence of trypsin at a molar ratio of 1:1000 (protease:protein) in the same buffer that was used in NMR studies. Free RPA70AB, binary complexes RPA70AB/Tag-OBD and RPA70AB/d(C)₈, and the ternary RPA70AB/Tag-OBD/d(C)₈ complex were incubated in the presence of trypsin and aliquots were removed at various time points. The protease activity was quenched by addition of SDS–PAGE loading buffer followed by boiling for 3 min. The digestion products were separated and visualized by 4–17% SDS–PAGE and Coomassie staining.

Tag-RPA pull-down assays

Purified Tag was bound to monoclonal antibody Pab101 adsorbed to protein G-agarose beads. Tag-bound beads were incubated with RPA or RPA70AB as indicated in the figure legends for 1 h at 4°C. After washing three times with washing buffer (30 mM HEPES–KOH (pH 7.9), 50 mM KCl, 7 mM MgCl₂, 0.25 % inositol, 0.05 % NP-40), the beads were resuspended in the SDS sample buffer and analyzed by SDS–PAGE and Western blotting with anti-RPA Mab70C and chemiluminescence. hRPA and RPA70AB binding to GST-Tag-OBd adsorbed to glutathione beads was assayed in a similar manner using anti-Tag Pab101.

In some experiments, increasing amounts of oligonucleotide dT₃₀, dT₁₅, or dT₈ were added to the washed Tag/RPA complexes on beads and incubated for 1 h at 4°C. After washing again with washing buffer, bound hRPA was visualized by SDS–PAGE,

immunoblotting with anti-RPA Mab70C (Kenny *et al*, 1990), and chemiluminescence.

ssDNA filter binding assays

Human RPA, yeast RPA, or hRPAy32C was incubated with 3 pmol of 5' ³²P-end-labeled ssDNA (dT₃₀) in binding buffer (30 mM HEPES–KOH (pH 7.9), 40 mM creatine phosphate, 7 mM MgCl₂, 4 mM ATP, 10 μM ZnCl₂) for 20 min at 37°C. Reactions were spotted on alkaline-treated nitrocellulose filters (McEntee *et al*, 1980). The filters were washed five times with wash buffer (30 mM HEPES–KOH (pH 7.9), 7 mM MgCl₂), dried, and analyzed by scintillation counting.

Native gel electrophoresis

Mobility shift reaction mixtures (15 μl) containing 3 pmol of 5' ³²P-end labeled ssDNA (dT₃₀) in binding buffer (30 mM HEPES–KOH (pH 7.9), 40 mM creatine phosphate, 7 mM MgCl₂, 4 mM ATP, 0.01 mM ZnCl₂) were pre-incubated with 2–6 pmol of RPA (human or yeast) as indicated in the figure legends at 25°C for 10 min. Tag was added as indicated in the figure legends for 15 min at 37°C. In some experiments, a purified monoclonal antibody was added 5 min later. The reaction products were analyzed after addition of loading buffer (2.5 % w/v Ficoll 400, 0.05 % w/v bromophenol blue, 0.05 % w/v xylene cyanol) and electrophoresis on 7.5 % polyacrylamide gels in 45 mM Tris, 45 mM boric acid, and 0.01 mM ZnCl₂ for 2 h at 100 V. The gel was dried and complexes were detected by autoradiography. Bound proteins were quantified by densitometry using IPLabGel.

Initiation of SV40 DNA replication

Monopolymerase assays (Matsumoto *et al*, 1990) were carried out as described recently (Arunkumar *et al*, 2005) except that 750 ng of Tag was used and RPA was varied as indicated in the figure legends. Reactions were assembled at 4°C and incubated at 37°C for 90 min. Reaction products were purified on G-50 Sephadex columns (Roche Diagnostics Corp., Indianapolis, IN) and precipitated with 100 % acetone. The washed and dried products were redissolved in loading buffer (45 % formamide, 5 mM EDTA, 0.08 % xylene cyanol FF, 0.08 % bromophenol blue) and resolved by 1.2 % alkaline (30 mM NaOH, 1 mM EDTA) agarose gel electrophoresis for 2–3 h at 100 V. The reaction products were visualized by autoradiography and quantified by scintillation counting.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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